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Full Length Research Paper

Laboratory studies on vegetative regeneration of the gametophyte of *Bryopsis hypnoides* Lamouroux (Chlorophyta, Bryopsidales)

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Vegetative propagation from thallus segments and protoplasts of the gametophyte of *Bryopsis hypnoides* Lamouroux (Chlorophyta, Bryopsidales) was studied in laboratory cultures. Thallus segments were cultured at 20°C, 20 µmol photons m^{-2} s^{-1}, 12:12 h LD); protoplasts were cultured under various conditions, viz. 15°C, 15 µmol photons m^{-2} s^{-1}, 10:14 h LD; 20°C, 20 µmol photons m^{-2} s^{-1}, 12:12 h LD; and 25°C, 25 µmol photons m^{-2} s^{-1}, 14:10 h LD. Microscope observation revealed that the protoplast used for regeneration was only part of the protoplasm and the regeneration process was complete in 12 h. The survival rate of the segments was 100% and the survival rate of protoplasts was around 15%, regardless of culture conditions. Protoplasts were very stable in culture and were tolerant of unfavorable conditions. Cysts developed at the distal end or middle portion of gametophytic filaments under low illumination (2 - 5 µmol photons m^{-2} s^{-1}), the key induction factor. Cysts formed several protoplast aggregations inside or the cyst as a whole detached directly from the matrix and all the units were able to develop directly into new gametophytes. Regeneration directly from protoplasts and thallus segments were also discovered in the field. A relatively completed life history of *B. hypnoides* is established with newly discovered propagation methods, namely protoplast regeneration.

Key words: *Bryopsis hypnoides*, protoplast, thallus segment, propagation method, life history.

INTRODUCTION

Bryopsis is globally distributed and has been identified in Japan, China (Yellow Sea coast), North Carolina, Southern Australia, Mediterranean Sea, Western North America and other regions. So far, studies of *Bryopsis* have been mostly focused on four aspects: (1) Ultrastructural observations and life history, contributing to knowledge of the fine structures of organelles and sexual reproduction (Hori and Ueda, 1967; Neumann, 1969; Urban, 1969; Burr and West, 1970; Morabito et al., 2003); (2) The wound healing or regeneration of the protoplast (Fritsch, 1935; Tatewaki and Nagata, 1970; Kobayashi and Kanaizuka, 1985; Menzel, 1988; Pak et al., 1991; Kim et al., 2001; Ye et al., 2005); (3) Natural products with important bio-activity against acquired immune deficiency syndrome (AIDS) opportunistic infections (Hamann et al., 1996; Becerro et al., 2001); and (4) other aspects, such as phototropic properties (Iseki et al., 1995a, 1995b; Ye et al., 2006).

The life history of *Bryopsis* is diplo-haplontic. Data on the life history of *B. hypnoides* Lamouroux were obtained in the 1970s in Banyuls, France (Neumann, 1970; Rietema, 1971a, b). There is also life history information on *Bryopsis plumosa* (Hudson) C. Agardh from Naples (Schussnig, 1930; Zinnecker, 1935; Rietema, 1970) and Banyuls (Rietema, 1970; Kermarrec, 1975) and *Bryopsis muscosa* Lamouroux (Kermarrec, 1975) from Banyuls. In those studies, gametophytes, either monoecious or dioecious, were reported to alternate with prostrate microthalli (sporophytes), which, after a variable period of dormancy, were able to follow two pathways: some isolates holo-carpically produced stephanokont zoospores, which

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Abbreviations: LD, Light/dark; **FFB,** free floating branch; **MA,** macrothallus.

developed into gametophytes; while in others, the gametophyte arose directly from sporophytes (Morabito et al., 2003). Populations of *B. plumosa* from Naples exhibit both patterns (Rietema, 1970).

Later works (Bartlett and South, 1973; Tatewaki, 1973; Kornmann and Sahling, 1976; Tanner, 1982; Rietema, 1969; 1972; 1975; Neumann, 1969; Richardson, 1982; Harper and Pienaar, 1985; Diaz-Piferrer and Burrows, 1974; Burr and West, 1970; Kermarrec, 1980; Brück and Schnette, 1997) revealed six different pathways from microthallus to macrothallus and two transitions needed require validation, viz. the steps from zygote to macrothallus and from gametangium to microthallus directly (Brück and Schnette, 1997).

Regeneration of protoplasts from *Bryopsis* has been studied for about 40 years. Protoplasts of *Bryopsis* can be easily obtained and regenerated into new individuals; however, there are only few studies that make use of a naturally occurring propagation method (Diaz-Piferrer and Burrows, 1974; Richardson, 1982; Brück and Schnetter, 1993) and pertinent information is still limited. Laboratory studies allow a better understanding of the life histories of Bryopsidaceae and have led to a taxonomic revaluation of this group (Morabito et al., 2003).

The present investigation is a contribution to the knowledge of the strategies of reproduction, growth pattern, as well as the life history of *Bryopsis* from the intertidal zone of Qingdao, China.

MATERIALS AND METHODS

Materials and culture

Gametophytic specimens of *B. hypnoides* were collected between August and November 2005 from the intertidal zone (35.35°N, 119.30°E, 20-50 cm depth) of Zhanqiao Wharf, Qingdao, China. Seawater was collected at the time of sampling using a pump placed 2 m depth under the water surface and filtered with nested plankton nets (200 µm net with 20 µm net inside). Collected water was autoclaved and made up into ES enriched seawater (Mclachlan, 1979). In the laboratory, the thalli were examined and those that were intact were isolated, washed several times with sterile seawater, sterilized with 1% sodium hypochlorite for 2 min and then rinsed with autoclaved seawater. The sterilized material was placed into a sterile aquarium (d = 40 cm, h = 30 cm) containing enriched seawater and maintained at 20°C, with 20 µmol photons m^{-2} s^{-1} provided by cool-white fluorescent tubes, with a photoperiod of 12:12 h LD.

Thallus segments and protoplast culture

For regeneration investigations, *B. hypnoides* thalli were sterilized again with 1% sodium hypochlorite for 2 min and rinsed with autoclaved seawater and the surface moisture was removed at once with 3 layers of absorbent paper. The clean algae were cut into segments (about 4 mm) and protoplasts were squeezed out with 8 layers of muslin into 10 ml Eppendorf tubes. Some of the initially extruded protoplasts were cultured in Petri dishes (d = 10 cm, h = 1.5 cm) containing autoclaved ES enriched seawater (1:30, v/v). The culture conditions included 3 combinations: 15°C, 15 µmol photons m^{-2} s^{-1}, 10: 14 h LD; 20°C, 20 µmol photons m^{-2} s^{-1}, 12:12 h LD; 25°C, 25 µmol photons m^{-2} s^{-1}, 14: 10 h LD. The survival rate of protoplasts was calculated after 1 week in culture. Ten Petri dishes were included in every treatment.

Longer gametophytic filaments were selected and cut into 1 cm long segments using a sapphire knife and cultured in Petri dishes (d = 6 cm, h = 1 cm) containing enriched ES seawater. 1200 segments were cultured; segments were evenly distributed in 80 Petri dishes. The culture condition was as follows: 20°C, 20 µmol photons m^{-2} s^{-1}, 12: 12 h LD. The induction experiment was performed under various low light conditions (1 - 10 µmol photons m^{-2} s^{-1}) combined other with conditions, viz. 15°C, 10:14 h LD; 20°C, 12:12 h LD; and 25°C, 14:10 h LD. All the culture media, including seawater used in the protoplasts culture, were changed once a day. Observations were also carried out every day.

For stress culture, one of the treatments was to break the primary envelop using a fine stainless needle 5 min after onset of culture; another was to culture the protoplasts under 20°C, 20 photons m^{-2} s^{-1} and 12:12 h LD without changing or adding any of the culture media during the whole experiment, leaving the protoplast in a high salinity environment caused by evaporation; the final treatment was culture of the protoplasts 4°C, 20 µmol photons m^{-2} s^{-1} and 12:12 h LD.

For induction culture, thallus regenerated plants were cultured at 20°C, 20 µmol photons m^{-2} s^{-1} and 12:12 h LD for 2 weeks, then transferred to a 4 µmol photons m^{-2} s^{-1} circumstance without changing other conditions. When the cysts evacuated their matrices or the protoplasts started to germinate, all of them were taken out and cultured under normal conditions (20°C, 20 µmol photons m^{-2} s^{-1}, 12:12 h LD).

Fixation and dyeing

For observation of the forming of the cell membrane, the fixative (ethanol: acetic acid: formalin: seawater = 3:1:1:3, v: v) and 0.125% commassie brilliant blue R250 (CBB R250) dye solution (ethanol: acetate acid: seawater = 40:5:55) were prepared. Protoplasts cultured in autoclaved seawater were fixed with the fixative at intervals. The fixation process in every treatment lasted for 30 min and then the material was dyed with 0.125% CBB R250 solution for 30 min.

Microscopy and photography

Observations were performed with a light microscope (Nikon, ECLIPSE 50i, Tokyo, Japan). Sizes of protoplasts were determined with an eyepiece micrometer and the number, including the survival rate, was counted randomly with an inverted microscope (ZEISS, HBO 50, Jena, Germany) at a magnification of × 200. Six microscope fields of view were selected randomly on each Petri dish and at least 3 Petri dishes were included in each batch under all culture conditions and all protoplasts in the field of view were included. Survival rate (%) = the number of protoplasts (or thallus segments) that developed into thalli one week after onset of culture/all the protoplasts observed (or all thallus segments cultured) × 100%. For stress culture, the viability and intactness of the protoplasts were routinely checked using neutral red and Evans blue one week after onset of culture.

RESULTS

Thallus regeneration

The wild mature thalli of *B. hypnoides* growing along the coastline of Yellow Sea were usually about 15 cm in

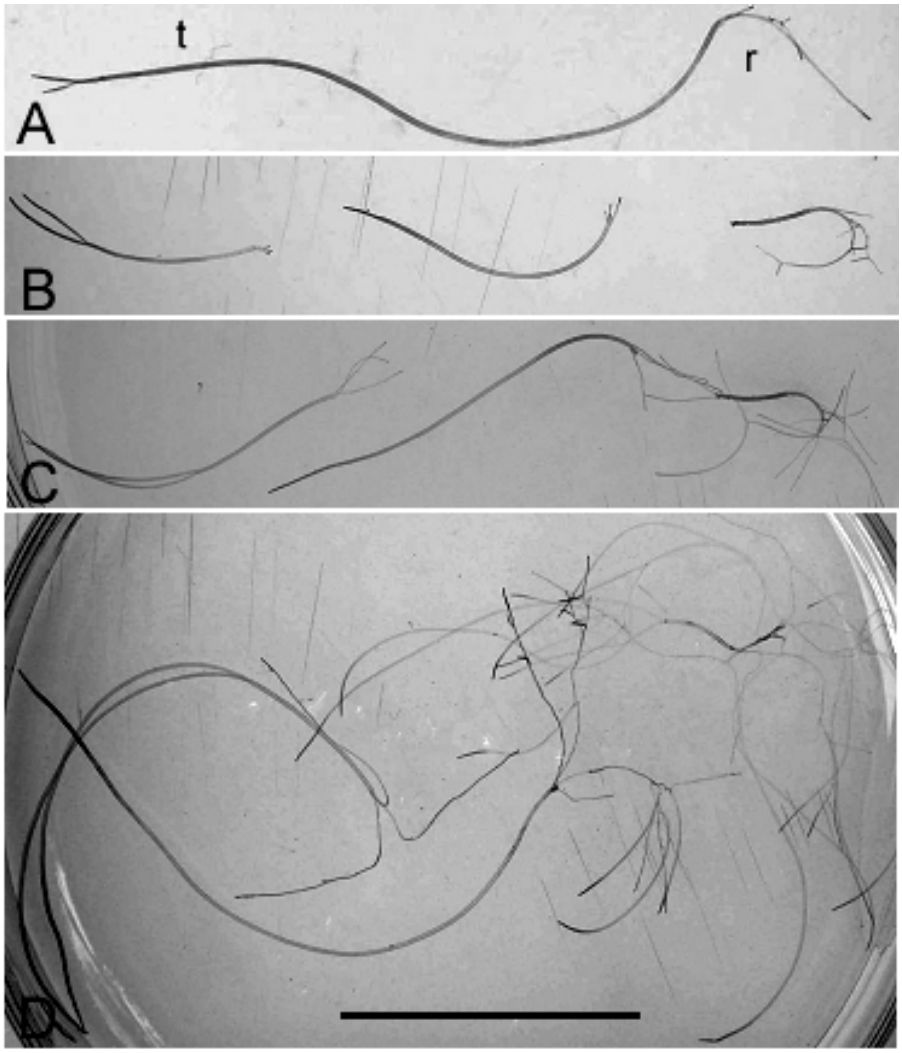

Figure 1. *Bryopsis hypnoides*: Thallus segment regeneration to the mature gametophytic phase. Scale bar, 2 cm. **(A)** A gametophytic filament selected for thallus segment regeneration. t, thallus; r, rhizoid. **(B)** Thallus segments showing newly developed thalli and rhizoid from the cuts. Segments were cut in sequence from the filament in B using a surgical blade, two days after the culture was initiated. **(C)** Developing gametophytic thallus, one week after culture initiation. **(D)** Developed gametophyte with many newly developed thalli branching from the rhizoid, 2 weeks after the culture was initiated.

length, appearing through the period of summer and autumn every year. The erect thalli are usually gracile, bearing rhizoids (Figure 1A). The thalli are fragile and easily broken by mechanical force. All segments survived and the regeneration process needed only a few days. The scar of the section healed within few hours after cutting and the ends protruded new erect thalli or rhizoid. After two days in culture, the newly generated thalli and rhizoid could be seen with the unaided eye (Figure 1B). Interestingly, rhizoids developed at the base of the segment, while a thallus formed distally (Figure 1C). Segments in culture always maintained this apico-basal orientation, indicating the existence of polarity. As a result of the rapid growth rate, the regenerated thallus developed into filaments in few weeks. Rhizoids developed into irregularly branched thalli that consisted of a tightly attached prostrate and detached filament (Figure 1D).

Aggregation process

The results from the experiment with the dye CBB R250 solution are shown in Figure 2. At the beginning of the culture, hydrophobic protein granules in blue color were distributed among the protoplasts (Figure 2A). The protein granules dispersed and moved to the outer layer

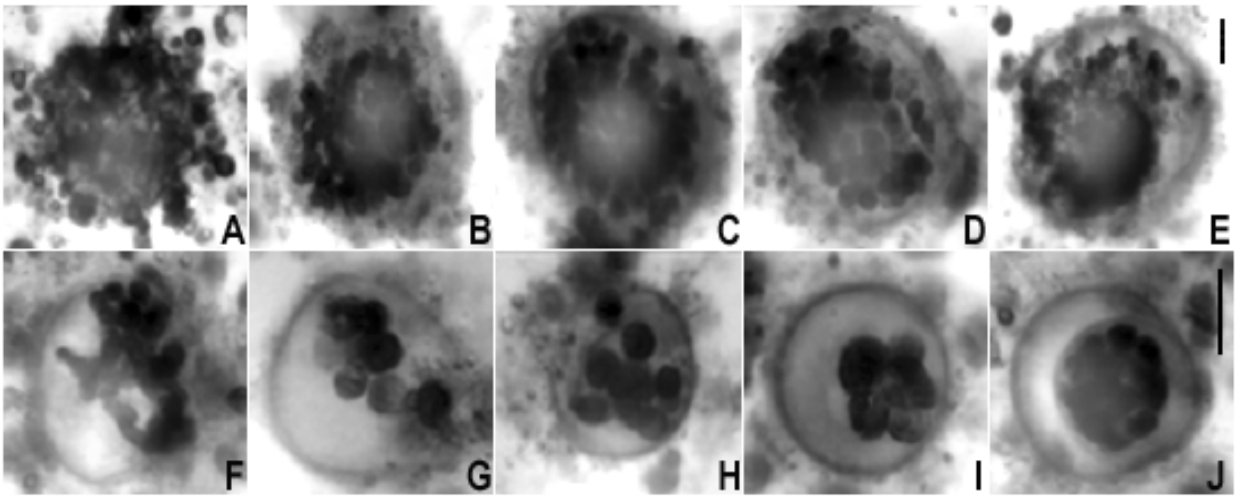

Figure 2. The formation of the cell membrane of sub-protoplast of *B. hypnoides*. Protoplasts cultured in autoclaved seawater were fixed with the fixative at intervals. The fixation process in every treatment lasted for 30 min and then the material was dyed with 0.125% CBB R250 solution for 30 min. Bar, 20 µm. **A** was fixed after 10 s onset of the culture. **B - J** were fixed every 40 min in turn and the total fixation period lasted for 6 h.

of the aggregate with the other organelles, such as chloroplasts, which were concentrated in the center (Figure 2B). Later, most of the protein granules dispersed and distributed around the aggregation and a faint pre-membrane (Figure 2C). With the passage of time, the pre-membrane turned clear and gradually became compact (Figures 2D - I). Finally, the second membrane formed at the periphery of the sub-protoplast (Figure 2J).

Culture of protoplasts

The culture conditions did not seem to affect the morphological expression or the development of the reproductive protoplasts. Most of the protoplasts dropped into seawater aggregated into protoplasts and the diameters of newly-formed protoplasts were from 10 to 100 µm (n > 110, each batch). Each protoplast produced a visible envelope within 2 to 10 min. Protoplasts adhered to the glass plates firmly and could not be dislodged when the culture medium was changed. About 60% of protoplasts were from 20 to 30 µm in diameter, regardless of culture conditions (Figure 3A). Protoplasts cultured at lower temperatures (15°C) aggregated slowly in comparison with those cultured under higher temperatures (20 and 25°C). However, survival rates were equivalent, with about 15% difference between temperatures (n > 600, each batch). One week later, the survivors began to generate (Figure 3B). The germlings of the protoplasts grew into thalli one month after the onset of culture.

Stress culture

Protoplasts with the protection of the newly-formed cell membrane and wall had high tolerance of unfavorable conditions (Figure 4). The newly-formed cell membrane was flimsy and easily broken by external mechanical forces. When seawater penetrated into the envelope through the interstices, the inner parts gathered again to quickly form a pre-membrane (Figure 4A). Changing the culture media periodically ensures suitable conditions for protoplast development; however, a few protoplasts did survive the stressful salinity conditions (Figure 4B). Six Petri dishes containing protoplasts were cultured under 4°C, 20 µmol photons m^{-2} s^{-1} and 12:12 h LD. Protoplasts were able to aggregate into protoplasts though 99.78% of them died (n = 1819) and others became dormant (Figure 4C).

Regeneration in the wild

In the field, an injured plant may persist through production of protoplasts and thallus segments within the shelter of the original cell wall (Figure 5). Although the primary cell wall plays an important role protecting the protoplasts from mechanical stresses or predators, it does restrict their further development (Figures 5A and B). Regenerated plants from broken thalli are readily found in the field (Figure 5C).

Induction experiment

Various cultivations at reduced light levels (1~10 µmol photons m^{-2} s^{-1}) were carried out and the best induction conditions were found to be 4 µmol photons·m^{-2} s^{-1}, 20°C and 12:12 h LD. Cyst induction and subsequent development are shown in Figure 6. The frequency of the new

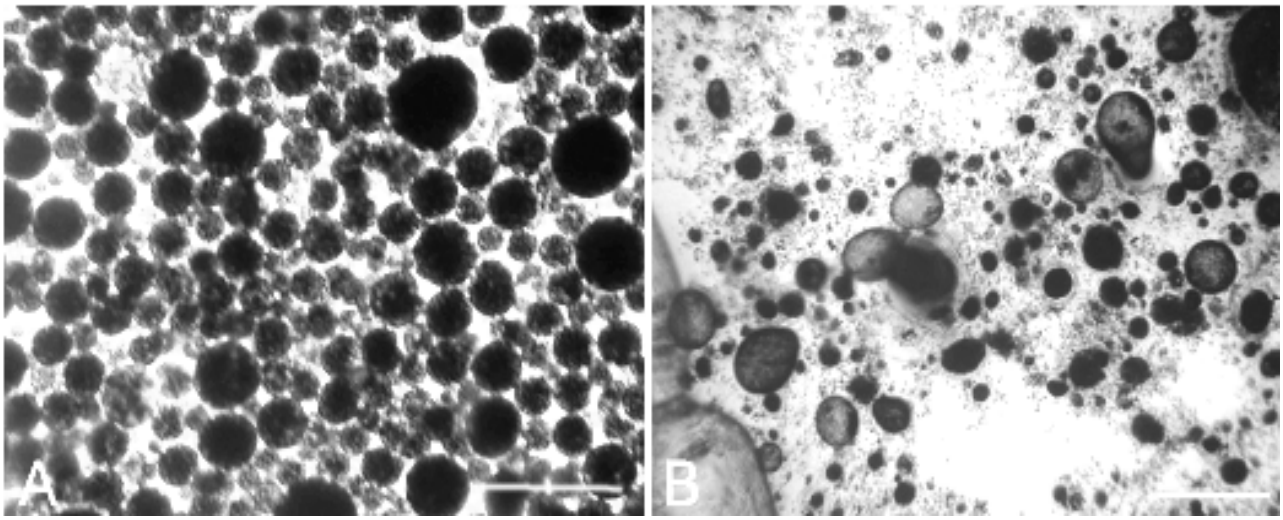

Figure 3. Protoplasts cultured under 20°C, 20 µmol photons m^{-2} s^{-1} and 12:12 h LD. Scale bar, 50 µm. **(A)** Aggregations appearing as rimless globes 2 min after the cultures was initiated. **(B)** Survivors starting to germinate, 1 week after the culture was initiated.

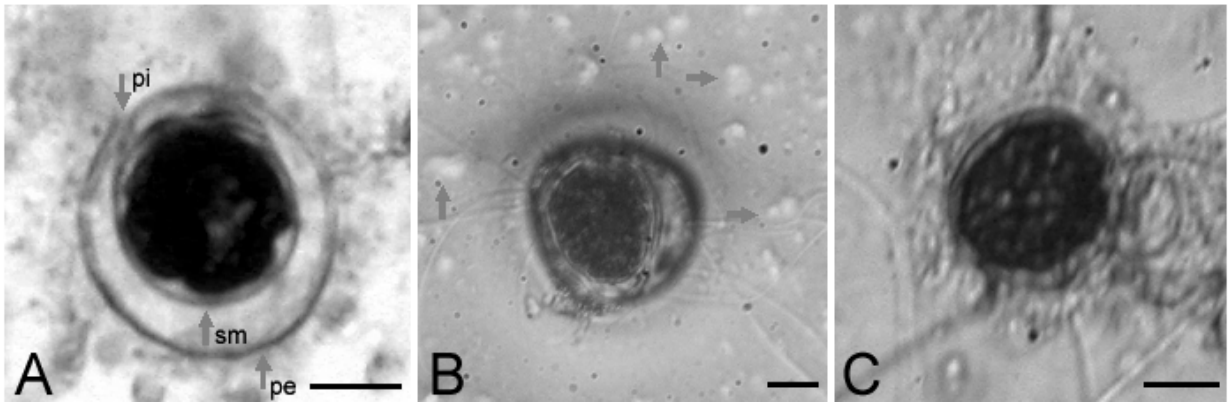

Figure 4. Protoplasts cultured under stress conditions. Scale bar, 30µm. **(A)** A protoplast covered with newly formed pre-membrane. pe, primary envelope; sm, second membrane. **(B)** A protoplasts cultured in a high salinity environment by without changing or adding culture media. Arrows show crystalloid salts from the seawater produced by evaporation, one week after onset of culture. **(C)** A protoplast cultured at 4°C, three months after the onset of culture. The other conditions were 20°C, µmol 20 photons m^{-2} s^{-1} and 12:12 h LD, except for the temperature in C.

form of propagation was less than 10% of the total population (n = 230, each batch) under the best induction conditions. Two weeks after, small capsule-like sacs called cysts formed at the distal ends or middles of erect thalli and rapidly became swollen (Figures 6A and B); this did not occur under normal culture conditions. Small cysts (sub-cysts) also developed from the primary cysts (Figure 6C). Some of the cysts developed from the distal end of the thalli expanded to 400 µm in length and 200 µm in width; in the cysts, newly-formed protoplasts developed into germlings (Figure 6D). Most of the cysts exited their matrixes (Figures 6E and F). As with the protoplasts, separate cysts developed into mature gametophytes several weeks later (Figures 6G, H and I).

DISCUSSION

Since *Bryopsis* is unicellular, it faces major problems when its cell wall is damaged. In multicellular algae, trauma is not as serious because the damage will extend only to those cells that have been ruptured. In *Bryopsis* and other siphonous algae, even the smallest tear in the cell wall and membrane risks spillage of all cell contents. Fritsch (1935) first reported that *Bryopsis* has a special clotting factor, similar to that of human blood, which is a combination of multiple small protein; when the cell wall is torn, these proteins collect at the opening and form a new cell membrane. Burr and Evert (1972) described wound healing of *B. hypnoides* with regards to thalli

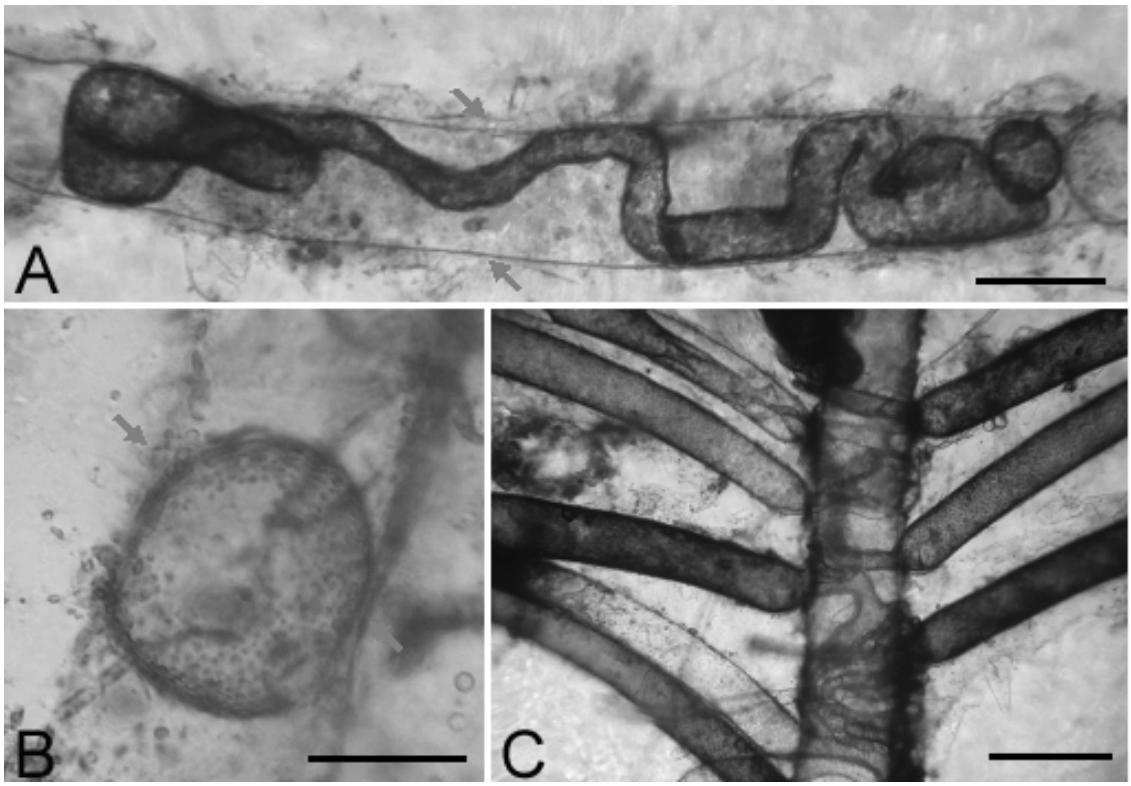

Figure 5. Regeneration of protoplasts and thalli in the field. Scale bars, 50 µm in A and B, 200 µm in C. **(A)** Protoplasts in a dead thallus. Arrows show the perimeter of the dead thallus. **(B)** A newly formed protoplast without germination. Arrows show the perimeter of the dead thallus. **(C)** New individuals derived from the branches, showing newly developed rhizoid at the bases of the segments.

regeneration from cut off segments and found whose wound-healing materials are proteinaceous substances. Kim and Klochkova (2005) recorded the aggregation process of protoplasts from *B. plumosa* using scanning electronic microscope and found a certain form of lectin that led to the protoplast formation. Clotting factors also aid *Bryopsis* in a form of secondary reproduction. If a piece of the plant is cut off by turbulence or grazing, clotting factors in the segment will seal the opening and the broken off segment can then drift and settle in a new location. In this way, *Bryopsis* may disperse. Our previous data showed that one of the protoplast-regenerated individual grew into thalli of 58 cm, 3-fold longer than the wild thalli and found that the total soluble proteins and the different ratios of chlorophyll *a* to chlorophyll *b* between the regenerated algae and wild ones were different (Ye et al., 2005).

Kim et al. (2001) pointed out that the survival rate of protoplasts was about 40%, much higher than in our experiment. The reasons behind the discrepancy may be as follows: (1) In our study, the survival rate were calculated one week after regeneration, while Kim et al. (2001) took readings 24 h after initiation; (2) The materials used belong to different species, *B. plumosa* and *B. bryopsis*, respectively. Ye et al. (2005) reported that on the diatom *Amphora coffeaeformis* (C. Ag.) Kützing could prevent protoplast germination; however, when the diatoms were removed, the protoplasts immediately geminated. More tolerant characters of protoplasts were found in the present study.

Light intensity is the key factor causing phase conversion. The cyst phase has a higher resistance to unfavorable conditions than the extruded protoplast because it is protected by the cell membrane and cell wall. It is not necessary for a cyst to undergo a dormancy period in order to germinate. Zygotes, on the other hand usually need some months to a year, or even more of dormancy to germinate (Rietema, 1969, 1970, 1971a, 1971b, 1975; Kermarrec, 1972; Diaz-Piferrer and Burrows, 1974; Jónsson, 1980; Morabito et al., 2003). The low light levels in the induction experiment are not suitable for long-term culture of the gametophytic filaments because there is no net photosynthetic gain. We believe that when a wild gametophyte of *B. hypnoides* is shaded in semi-darkness the filaments will change their life phase to produce cysts. Cysts can exit the filaments and disperse to an illuminated site. Illumination stress is common and could bring about life cycle phase shifts in *B. hypnoides*.

On the basis of our work and earlier studies, we are able to propose a relatively complete life cycle for *B. hypnoides*. Other parts except for Parts A and B have been described by Brück and Schnetter (1993, 1997).

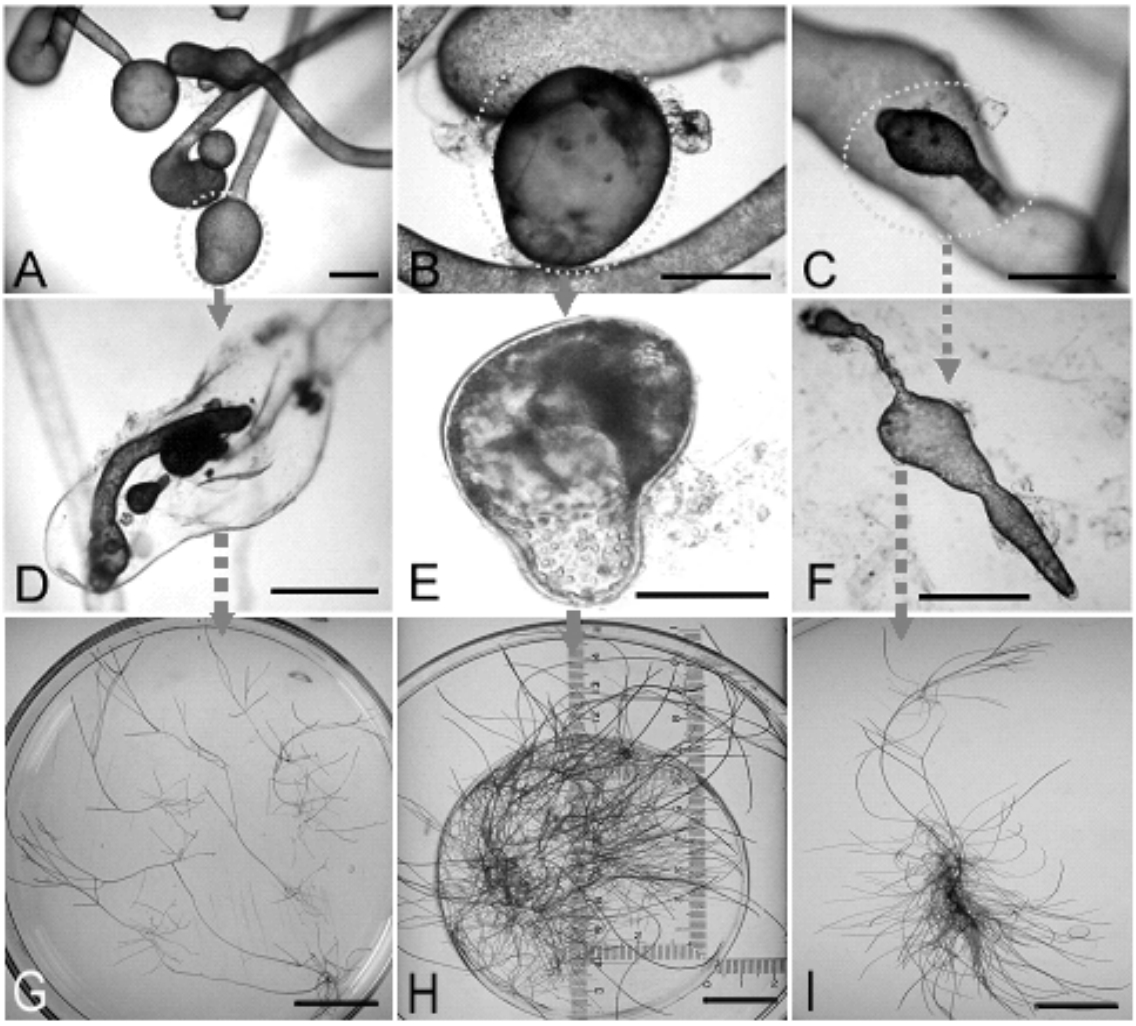

Figure 6. Induced cysts and their development. Scale bars, 100 μm in A, B, C, D, E and F; 2 cm in G, H and I. The dashed circles enclose developing under inducting conditions; arrows indicate the derivation and development of the cysts. **(A)** Cysts developing at the distal end of a thallus. Some cysts bear sub-cysts. **(B)** A cyst formed in the middle of the filament. **(C)** A sub-cyst developing from a mother cyst. **(D)** Protoplasts generating in a dead giant cyst. **(E)** A swelling cyst. **(F)** A germinating sub-cyst. **(G)** Plants regenerated from protoplasts derived from a giant cyst, 2 weeks after germination. **(H)** Gametophytic filaments regenerated from a cyst derived from the middle of a filament, one month culture after induction. **(I)** A gametophytic plant regenerated from a sub-cyst, 3 weeks after induction.

The direct development of free floating branch (FFB) con-firms the importance of the macrothallus (MA) generation for the survival of *Bryopsis* species. Further experiment needs to be conducted to validate the variation from MA to protoplast (SP) directly.

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